REMARKS

Upon entry of the foregoing Amendment, Claims 1-10 and 12-13 will remain pending in the application. Claims 8-9 have been withdrawn from consideration. Claim 11 has been canceled; and Claims 12-13 have been added. Support for the newly added Claim 12 can be found in the specification, at least on page 7, lines 26-31. Support for the newly added Claim 13 can be found in the specification, at least on page 9, Example 1 (the specification recites that "A Pseudomomonas 7A glutaminase-asparaginase modified with polyethylene glycol (DE 41 40 003 A1, WO 94/13817 A1 and WO 02/31498 A2) was used as the glutaminase"). These changes do not introduce new matter, and their entry is respectfully requested.

In the Office Action of April 15, 2009, the Examiner set forth a number of grounds for rejection. These grounds are addressed individually and in detail below.

Claims Rejections Under 35 U.S.C. § 112 (Written Description)

Claim 11 stands rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement for the reasons set forth on pages 2-3 of the outstanding Office Action. Applicants respectfully traverse the rejection. In order to expedite the prosecution of this case, Claim 11 has been canceled.

In view of the foregoing, the grounds for this rejection have been obviated and the withdrawal of the rejections under 35 U.S.C. § 112, first paragraph is respectfully requested.

Claims Rejections Under 35 U.S.C. § 103

Claims 1-5, 7, 10 and 11 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Leskovar et al. (WO 89/09620 of PCT/EP89/00403, which has the English language equivalent document U.S. Patent Publication No. 2002/0094542 which is a 35 U.S.C. § 371 National stage of the priority document) (hereinafter "Leskovar"); Claims 1-5, 7, 10 and 11 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Leskovar, in light of support by Sugiura et al. Gann, 1982 (hereinafter "Sugiura"); Claims 1-7 and 10 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Leskovar as applied to Claims 1-5, 7 and 10, and further in view of Housmen et al. (U.S. Patent 6,200,754) (hereinafter "Housmen"); Claims 1-3, 5-7, 10 and 11 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Sugiura; and Claims 1-7, 10 and 11 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Sugiura as applied to Claims 1-3, 5-7, 10 and 11 above, and further in view of Roberts et al. (J.Gen.Virology, 1991), (hereinafter "Roberts"). Applicants respectfully disagree.

Again, to establish a *prima facie* case of obviousness of a claimed invention, all the claim limitations must be taught or suggested by the prior art. *In re Royka*, 490 F. 2d981, 180 USPQ 580 (CCPA, 1074).

The present Claim 1 is directed to a pharmaceutical composition for cancer therapy consisting essentially of: a) at least one compound having glutaminase activity; b) at least one antineoplastic agent selected from the group consisting of platinum complexes and anthracyclines; and c)at least one of carrier substances, auxiliary substances, and pharmaceutical injection media.

In contrast, Leskovar only describes a medicament which comprises (1) antibodies or conjugates of antibodies and cytotoxic agents *(i.e.*, component A) and (2) activators of effector cells (*i.e.*, component B). The cytotoxic agents include anthracyclines such as doxorubicin and daunomycin (Leskovar, paragraph [0023]). The conjugates of antibodies include antibodies conjugated to asparaginase or glutaminase (Leskovar, paragraph [0192]).

Again, Leskovar does not teach or suggest using unconjugated anthracyclines in a pharmaceutical composition, as recited in instant Claim 1. In fact, by including activators of effector cells as one of the two key ingredients of the claimed invention, Leskovar **teaches away** from a composition that does not contain activators of effector cells. For this reason alone, instant claim 1 is patentable over Leskovar.

In Response to Examiner's Arguments

The Examiner states on page 4, 2nd paragraph of the Office Action that the Examiner shall use the "broadest **reasonable** interpretation consistent with the specification" (emphasis added). Applicants submit that Leskovar does not teach or suggest at least one compound having glutaminase activity as defined in Claim 1. Additionally, the anthracycline-antibody-conjugates as described in Leskovar would not be considered by one ordinary skill in the art as derivatives of anthracyclines. Leskovar only mentions **conjugates, not derivatives**. However, conjugates and derivatives are two different terms.

Derivatization is a technique used in chemistry which transforms a chemical compound into a product of similar chemical structure, called a derivative (see Exhibit 1, Wikipedia). Generally, a specific functional group of the compound participates in the derivatization reaction and transforms the educt to a derivate. Therefore, the structure and properties of the original compound are altered.

Conjugation of small molecules like anthracyclines to antibodies is resulting in new antibody compounds (Exhibit 2, About.com), but not in a derivatization of anthracyclines. The chemical structure of the new compounds as well as the size of the new antibody molecules differs substantial from anthracyclines and/or anthracycline derivates respectively. Also the mechanism of action of the new compound differs to that of anthracyclines, *e.g.* the new antibody compound is much bigger than the conjugated anthracyclines and cannot cross the cell membrane to inhibit DNA and RNA synthesis by intercalating between base pairs of the DNA/ RNA strand which is a mechanism of action of anthracyclines.

Further, the Examiner states that in Leskovar, the anthracydine would be simply derivatized with an antibiotic group. This statement is inconsistent because the compositions taught by Leskovar are anthracycline-**conjugated** with an antibody. As recited in paragraph [0140] of Leskovar, cytotoxic drugs, such as doxorubicins are **not derivatized** with an antibiotic group but rather **conjugated** with an antibody.

The Examiner then further states on page 5 of the Office Action that "however compositions are defined by the structure of their components and if the composition is physically similar or indeed the same, obviously it must have the same properties including mechanism." Nonetheless, Applicants submit that the components described in Leskovar are basically different from the components used according to the present claimed invention.

According to the therapeutic approach of Leskovar, Leskovar used several different types of antibodies and antibody conjugates respectively to affect the immune system (cf. Abstract: "... a component A, eliminating suppressor T-cells in a specific or non specific way, and a component B, activating effector cells"). Applicants submit that the therapeutic effect described by Leskovar resulting from an immunotherapy by using antibodies that are completely

different from the claimed chemotherapeutic compound *e.g.* using anthracyclines. Furthermore, enzymes of Leskovar (*e.g.* glutaminase) are **not used to treat cancer** but is used as an add-on to the antibodies and to circumvent the eventual problems associated with the reticulo endothelic system (RES)-toxicity of immunotoxins. (See [0192] of Leskovar).

The Examiner further alleges that "the art is replete with references where anthracydine conjugated to antibodies continue to be considered "anthracyclines" as supported by Angelucci et al." The Examiner's contention is respectfully traversed. Angelucci clearly differentiates anthracydine conjugates, *i.e.* anthracydine conjugates with carriers such as antibodies and anthracyclines. Angelucci explicitly mentions that anthracydine conjugates (*i.e.* anthracycline-antibody conjugates) have higher potencies than anthracyclines, which clearly shows that Angelucci uses these two terms differently and not synonymously (see Abstract of U.S. 5,776,458).

As mentioned above, anthracyclines are completely different from antibodies-anthracycline-conjugates. Leskovar himself differentiates these terms. Leskovar states in paragraph [0023] that the distinction between component A an AB-conjugate (see U.S. 2002/0094542 A1, page 2, left column, last-but-one-line to right column, 3rd line). Leskovar mentions anthracyclines and AB-conjugates of anthracyclines as separate entities. Insofar the interpretation of the Examiner is even contrary to the teaching of Leskovar.

Further, the Examiner misinterprets paragraph [0026] of Leskovar in the outstanding Office on page 6, line(s) 18. Leskovar on paragraph [0026] describes the induction of a tolerance of the immune system against conjugates with xenogeneic proteins and does not imply the use of the combination of different components A. The purpose of xenogeneic proteins is not explained by Leskovar, but is probably to increase the immunogenicity/xenogeneity of the

conjugates, which could result in a stronger immune reaction and a more efficient elimination of the targeted cells by cells of the RES.

Therefore Leskovar teaches the use of glutaminase as **solely** an immunotherapeutical substance. Furthermore Leskovar teaches in paragraph [0195], that "the enzymatic therapy, based on a systemic administration of the enzyme asparaginase or arginase (per se, not as conjugate), is non-selective and non-localized."

As a result, a person of ordinary skill in the art would not have a reasonable expectation of success in view of achieving the claimed invention comprised of the enzyme. The synergistic effect in using anthracyclines and glutaminase of the present claimed invention was completely unexpected and not obvious.

In regards to the arguments presented by the Examiner related to Sugiura and Robert, neither Sugiura nor Robert mentions a pharmaceutical composition comprising both, at least one compound having a glutaminase activity and at least one anti-neoplastic agent selected from the group consisting of platinum complexes and anthracyclines. In the publication of Sugiura, glutaminase alone does not show a significant anti-tumoral effect (see the tumor index of 0.91 in Table II). Furthermore the glutaminase in the present claimed invention is not a cytotoxic substance like Pt-complexes and anthracyclines, it works as an enhancer substance. Therefore, it is not obvious in light of Leskovar and Sugiura to combine glutaminase and cytoxic agents like Pt-complexes and anthracyclines.

Lastly, Housman does not cure the deficiency of Leskovar, Sugiura and Robert.. Houseman is cited for its teachings on mitomycin C and *cis*-platinum. Houseman does not teach or suggest using unconjugated anthracyclines in a pharmaceutical composition, nor does it teach or suggest a composition consisting essentially of a compound having glutaminase activity and unconjugated anthracyclines, as recited in Claim 1 of the instant application.

In view of foregoing, Applicants respectfully submit that Leskovar, Sugiura, Housmen and Roberts, individually or in combination, do not render Claim 1 obvious because they fail to teach or suggest all the claim limitations. Applicants further submit Claims 2-7, 10 and 12-13 are patentable over Leskovar, Sugiura, Housmen and Roberts because they depend from Claim 1 and recite additional patentable subject matter.

Newly Added Claim 13

Claim 13 is directed to a pharmaceutical composition for cancer therapy consisting essentially of: a) at least one compound having glutaminase activity **consisting of a tetramer composed of four subunits with a molecular weight of approximately 35 KDa**; b) at least one antineoplastic agent selected from the group consisting of platinum complexes and anthracyclines; and c) at least one of carrier substances, auxiliary substances, and pharmaceutical injection media.

Example 1, on page 9 of the present specification recites that "A Pseudomomonas 7A glutaminase-asparaginase modified with polyethylene glycol (DE 41 40 003 A1, WO 94/13817 A1 and WO 02/31498 A2) was used as the glutaminase". Page 3, lines 5-6 of WO 94/13817 A1 recites "*Pseudomonas* 7A glutaminase-asparaginase is composed of four identical subunits with

a molecular weight of approximately 35,000". It is well known in the art that glutaminase-asparaginase and glutaminase consist of four subunits (tetramer) for activity (see, Exhibit 3, Holcenberg, et al. 1976, *J. Biol. Chem* 251:5375-5380).

In contrast, Leskovar generally describes a medicament which comprises (1) antibodies or conjugates of antibodies and cytotoxic agents (*i.e.*, component A) and (2) activators of effector cells (*i.e.*, component B). The cytotoxic agents include anthracyclines such as doxorubicin and daunomycin (see paragraph [0023]). Both components consist of antibody-substance-conjugates, where the substances either eliminates (help in elimination, respectively) suppressor cells (in case of component A) or activates the effector cells (in case of component B).

Further, Leskovar discloses the conjugates of antibodies to asparaginase or glutaminase (see paragraph [0192]). As noted in the Response filed by Applicants on July 15, 2008, the molecular weight of an antibody is between about **50 and 200KDa**, depending on the Ig type. While an anthracycline is a small molecule (approx. 0.5k Da), an antibody-anthracycline-conjugate (approx. 50-200 kDa) is essentially a protein with a severalfold larger molecular mass and altered properties, in particular with respect to the ability to cross borders like membranes and to translocate between different tissues.

Therefore, Leskovar does not teach or suggest at least one compound having glutaminase activity **consisting of a tetramer composed of four subunits with a molecular weight of approximately 35 KDa** as recited in Claim 13.

Sugiura, Housmen and Roberts do not cure the deficiency of Leskovar since neither Sugiura, Housmen nor Roberts mentions a pharmaceutical composition for cancer therapy consisting essentially of at least one compound having glutaminase activity **consisting of a tetramer composed of four subunits with a molecular weight of approximately 35 KDa**, combined with at least one antineoplastic agent and at least one of carrier substances, auxiliary substances, and pharmaceutical injection media as recited in Claim 13.

Therefore, new Claim 13 is patentable over Leskovar Sugiura, Housmen and Roberts.

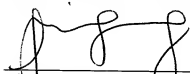
CONCLUSION

All of the stated grounds of rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding rejections and that they be withdrawn. It is believed that a full and complete response has been made to the outstanding Office Action and, as such, the present application is in condition for allowance.

If the Examiner believes, for any reason, that personal communication will expedite prosecution of the application, the Examiner is invited to contact Applicants' counsel, Ping Wang, M.D. (Reg. No. 48,328), at 202.842.0217.

Respectfully submitted,

MORRIS, MANNING & MARTIN, LLP



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Attachments:
EXHIBIT 1
EXHIBIT 2
EXHIBIT 3

Derivative (chemistry)

Exhibit 1

From Wikipedia, the free encyclopedia

In chemistry, a **derivative** is a compound that is formed from a similar compound *or* a compound that can be imagined to arise from another compound, if one atom is replaced with another atom or group of atoms.[1] The latter definition is common in organic chemistry. In biochemistry, the word is used about compounds that at least theoretically can be formed from the precursor compound.[2]

Chemical derivatives may be used to facilitate analysis. For example, melting point (MP) analysis can assist in identification of many organic compounds. A crystalline derivative may be prepared, such as a semicarbazone or 2,4-dinitrophenylhydrazone (derived from aldehydes/ketones), as a simple way of verifying the identity of the original compound, assuming that a table of derivative MP values is available.[3] Prior to the advent of spectroscopic analysis, such methods were widely used.

See also

- Derivatization

References

1. ^ "Definition of Derivative". Chemicool. 2007-09-18. http://www.chemicool.com/definition/derivative.html. Retrieved 2007-09-18.
2. ^ *Oxford Dictionary of Biochemistry and Molecular Biology*. Oxford University Press. ISBN 0-19-850673-2.
3. ^ Williamson, Kenneth L. (1999). *Macroscale and Microscale Organic Experiments, 3rd ed.*. Boston: Houghton-Mifflin. pp. 426–7. ISBN 0-395-90220-7.

Exhibit 2

Conjugate Definition

By Anne Marie Helmenstine, Ph.D., About.com

See More About: conjugate chemistry definitions glossary compounds acids and bases

Definition: In chemistry, a conjugate refers to a compound formed by the joining of two or more chemical compounds or the term conjugate refers to an acid and base that differ from each other by a proton.

Return to the Chemistry Glossary Index

A B C D E F G H I J K L M N O P Q R S T U V W X Y Z

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Physical Properties of Antitumor Glutaminase-Asparaginase from *Pseudomonas 7A**

(Received for publication, March 22, 1976)

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Glutaminase-asparaginase from *Pseudomonas 7A* appears to have four subunits with a molecular weight of 36,000 ± 500 by sedimentation equilibrium in 5.9 M guanidine HCl and 34,000 by amino acid analysis. Analytic sedimentation equilibrium of the native enzyme showed a molecular weight of 140,000 ± 3,300 with no signs of association or dissociation. Moving boundary and zone sedimentation in buffer showed normal behavior with sedimentation coefficients of 7.92 and 7.75 S, respectively. In contrast, the enzyme appeared to polymerize during zone sedimentation when the initial protein concentration was greater than 1 mg/ml and the buffer contained asparagine, glutamine, or 5-diazo-4-oxonorvaline.

An extension of our method for active enzyme sedimentation is described which utilizes the changes in absorption during hydrolysis of asparagine by low concentrations of enzyme. Polymerization was not seen under these conditions.

The purification and properties of a new glutaminase-asparaginase from *Pseudomonas 7A* have been reported recently (1). This enzyme has a higher ratio of glutaminase to asparaginase activity, a much longer plasma half-life, and greater antitumor activity than *Acinetobacter* glutaminase-asparaginase. A detailed study was undertaken to ascertain whether differences in physical properties of these two enzymes as well as others could account for the biologic differences. The physical properties of *Acinetobacter* glutaminase-asparaginase were described in a previous report (2). This paper reports the behavior of the *Pseudomonas 7A* enzyme during zone, active enzyme, and equilibrium sedimentation in the analytical ultracentrifuge. This enzyme appears to polymerize at high protein concentration but only in the presence of substrate. An extension of our method for active enzyme sedimentation is described which utilizes the change in absorption during hydrolysis of asparagine by low concentrations of enzyme (2, 3).

EXPERIMENTAL PROCEDURE

Crystalline bovine albumin was purchased from Miles Laboratories; alcohol dehydrogenase and catalase from Boehringer Mannheim; FC-43 from Minnesota Mining and Manufacturing; and Nessler's compound from Harleco (#64992). Ultrapure guanidine HCl from Heico was dissolved in 0.01 M sodium phosphate buffer, and the pH was adjusted to 7 with NaOH. Density of this solution was 1.1423 measured with a 10-ml pyknometer at 20°. This density indicates that the guanidine solution was 5.9 M.

Glutaminase-asparaginase from *Pseudomonas 7A* was isolated and purified by Dr. Joseph Roberts (1). The enzyme used in these studies was from a single batch, which was homogeneous on disc gel electrophoresis and had a specific glutaminase activity of 165 IU/mg of protein prior to lyophilization. Reconstituted enzyme had a specific activity of about 100 IU/mg of protein. The enzyme activity was not inhibited by sodium azide. Enzyme activity was assayed by ammonia formation from asparagine. Activity with 5-diazo-4-oxonorvaline was monitored spectrophotometrically (4). Protein concentration was determined by ultraviolet absorption ($E^{1\%}_{1cm}$ = 9.8) (1).

Gel filtration was performed on a 100 to 200 mesh fraction of Bio-Gel P-200. A chromatronix column (1.27 × 109 cm) was pumped with upward flow at about 2 ml/h with 0.01 M sodium phosphate buffer, pH 7.3 to 7.4, containing 0.2 M NaCl and 3 mM sodium azide. Protein standards were catalase, bovine serum albumin, and alcohol dehydrogenase. Elution of alcohol dehydrogenase and the glutaminase-asparaginase was monitored by enzyme activity as well as absorbance at 280 nm.

Amino acid analysis was performed by AAA Laboratories, Seattle, WA, on samples hydrolyzed for 24 and 96 h in 6 N HCl at 110°. Additional samples were hydrolyzed in base for tryptophan determination (5). Minimum molecular weight from amino acid analysis was calculated by the method of Delaage (6).

Ultracentrifuge studies of active enzyme, sedimentation velocity, and sedimentation equilibrium were performed as previously described with a Beckman-Spinco model E analytic ultracentrifuge equipped with an ultraviolet absorption scanning system linked to PDP-12 computer (2, 3). Photographic plates of sedimentation equilibrium runs were read by an automatic plate reading device similar to that described by DeRosier *et al.* (7). Partial specific volume of the enzyme was calculated from the amino acid composition to be 0.735 ml/g. For experiments in guanidine hydrochloride, the apparent partial specific volume was calculated by the method of Lee and Timasheff (8); the result was ϕ' = 0.735 ml/g.

* This investigation was supported by United States Public Health Service Grants CA 11881, GM 13401, CA 08748, and CA 15860.

‡ Career Development Awardee, National Cancer Institute, CA 25976. Current address, Department of Pediatrics, Medical College of Wisconsin, Milwaukee Children's Hospital, Milwaukee, Wis. 53233.

RESULTS

Subunit Molecular Weight—The amino acid analysis on duplicate samples hydrolyzed 24 and 96 hours is shown in Table I. No cysteine was detected. A subunit molecular weight was calculated by a method which minimizes derivation of each amino acid from integers (6). The best molecular weight between 30,000 and 45,000 was 34,000 g/mol when reached at increments of 500 g/mol. The values for the individual amino acids with this subunit molecular weight are shown in the second column of Table I.

Equilibrium sedimentation was performed on enzyme dialyzed for 3 days at room temperature in 5.9 M guanidine HCl and 0.01 M sodium phosphate buffer (pH 7). The protein at 0.45, 0.67, and 1.35 mg/ml was sedimented at 36,000 rpm and 20° for 47 hours. Analysis of the Rayleigh plates is shown in Fig. 1. A subunit molecular weight of 36,000 ± 500 g/mol was obtained from the values at concentrations greater than 1.5 fringes extrapolated to zero concentration (9). A portion of the guanidine-treated enzyme was dialyzed exhaustively in 0.01 M sodium phosphate buffer. Sixty-four per cent of the original enzyme activity was recovered indicating the denaturation is reversible.

Molecular Weight of the Native Enzyme—Gel filtration on a calibrated Bio-Gel P-200 column gave a symmetrical single peak of enzyme activity and protein absorbance. Molecular weight from these experiments was 104,000 g/mol. One experiment with enzyme preincubated and run in buffer containing 200 mM asparagine showed a symmetrical peak with similar elution volume.

For sedimentation equilibrium experiments to determine the molecular size of the native enzyme, it was dialyzed overnight at 5° in 0.01 M sodium phosphate buffer (pH 7.4) containing 0.2 M NaCl and 3 mM sodium azide. The initial concentrations of enzyme were 0.32, 0.65, and 0.97 mg/ml. The Yphantis cell was centrifuged at 16,000 rpm for 23 h and then at 14,000 rpm for an additional 67 h. Fig. 2 shows a plot of the molecular weight of each channel at various fringe displacements. The figure shows that the enzyme is homogeneous and does not associate or dissociate. Analysis of these data gave a molecular weight and root mean square error of 139,100 ± 900 g/mol. A similar pattern was seen in analysis of the Rayleigh plates from the run at 16,000 rpm and equilibrium runs at 14,000 rpm in the same buffer containing either 20 mM aspartic acid or 3% sucrose (Table II). The average molecular weight from all the data was

TABLE I
Amino acid composition

Amino acid	Residues/1,000[a]	Mol/Subunit[a]
Aspartate	124	39.4
Threonine	55	17.2
Serine	72	22.8
Glutamate	105	33.4
Proline	28	9.0
Glycine	82	25.9
Alanine	108	34.1
Valine	100	31.6
Methionine	22	6.9
Isoleucine	54	17.1
Leucine	79	24.9
Tyrosine	22	6.9
Phenylalanine	18	5.8
Histidine	15	4.8
Lysine	68	21.6
Arginine	38	12.0
Tryptophan	10	3.2

[a] Values are calculated from duplicate analyses at 24 and 96 h. Values for threonine and serine were extrapolated to zero time. Values from the 96-h hydrolyses were used for valine, isoleucine, leucine, and lysine. Tryptophan was calculated from its ratio to histidine after base hydrolysis.

[b] Subunit molecular weight taken as 34,000 g/mol as calculated by the method of Delaage (6).

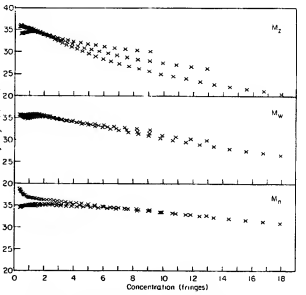

FIG. 1. Molecular weight distribution in guanidine HCl. Data presented are computer-generated plots from analysis of a Rayleigh plate taken at 47 h at 36,000 rpm and 20°. The solvent was 5.9 M guanidine HCl and 0.01 M sodium phosphate (pH 7). Enzyme had been dialyzed 3 days in the same solvent. The sectors contained 0.45, 0.67, and 1.35 mg of protein/ml. M_z, M_w, and M_n represent the number, weight, and Z-average molecular weight.

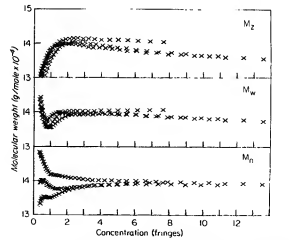

FIG. 2. Molecular weight distribution of native enzyme. Data presented are computer-generated plots from analysis of a Rayleigh plate taken after centrifugation at 16,000 rpm for 23 h and 14,000 rpm for an additional 67 h. Buffer was 0.01 M sodium phosphate (pH 7.4) containing 0.2 M NaCl and 3 mM sodium azide. The initial concentration of enzyme was 0.32, 0.65, and 0.97 mg of protein/ml. The abbreviations used are explained in the legend to Fig. 1.

140,900 ± 3,300 g/mol. These results, together with the subunit molecular weight of 36,000 ± 500 g/mol, indicate that the enzyme is most likely a tetramer with four subunits.

Sedimentation Velocity and Active Enzyme Sedimentation—A moving boundary experiment was performed with 0.4, 0.6, and 0.8 mg/ml of protein in 0.01 M sodium phosphate buffer (pH 7.4) and 0.2 M NaCl. Equivalent boundary calculations gave an extrapolated sedimentation coefficient at zero concentration $s^0_{20,w}$, of 7.92 ± 0.08 S.

The results of zone sedimentation in the same buffer containing 3% sucrose are shown in Table III. The sedimentation coefficient obtained from peak absorbance was slightly lower than from equivalent boundary calculations, but the zones were symmetrical and showed no evidence of dissociation of the tetramer. Average $s_{20,w}$ was 7.59 and $\bar{s}_{20,w}$ was 7.75 S.

At 25°, the enzyme hydrolyzes 0.1 mM 5-diazo-4-oxonorvaline at a 0.06% the maximal rate of asparagine hydrolyses at 37°. As described previously, sedimentation of a zone of enzyme through a solution of 5-diazo-4-oxonorvaline can be used to calculate the sedimentation coefficient of the active enzyme species (2, 3). Four such experiments at 60,000 rpm with the same buffer containing 80 μM 5-diazo-4-oxonorvaline gave higher sedimentation coefficients than the zone sedimentation velocity experiments in buffer alone. Plots of log X displacement with time were not linear but showed a more rapid displacement with increasing time. Results of one of these experiments (#2009) are presented in Table III. A single run (#2011) at 52,000 rpm did not show this anomalous behavior.

To investigate this apparent polymerization further, 5-μl zones of 1.2 mg/ml of protein were sedimented in buffer containing asparagine and glutamine. These experiments showed the same increase in the rate of sedimentation with increasing time. Fig. 3 shows a plot of displacement of log X with time for zones in buffer and 100 mM asparagine. An acceleration of sedimentation rate was apparent after 8 min in the experiment with 100 mM asparagine. With 20 mM asparagine, the acceleration was not seen until 26 min after the rotor reached 52,000 rpm. Fig. 4 shows the absorbance patterns from these experiments from scans taken 26 min after the rotor reached speed. The lower pattern shows the symmetrical pattern seen with buffer alone. The middle pattern, from a run in 20 mM asparagine, shows material sedimenting faster than the main peak. The upper pattern, from a run in 100 mM asparagine, shows a much smaller main peak and more faster-sedimenting material. An identical pattern was seen from a zone experiment in 100 mM glutamine.

This polymerization of the protein in the presence of substrate might explain the long biologic half-life by decreas-

TABLE II

Molecular weight of native enzyme

Molecular weight calculated from equilibrium sedimentation experiments in 0.01 M phosphate buffer (pH 7.4) containing 0.2 M NaCl and 3 mM sodium azide.

Buffer additives	Molecular weight	RMS error[a]
None	139,000[b]	3,500
	139,100	900
20 mM Aspartate	142,700	1,100
3% Sucrose	145,800	900

[a] Root mean square error.
[b] Equilibrated at 16,000 rpm; other experiments at 14,000 rpm.

TABLE III

Comparison of sedimentation coefficients

Zero time is when the rotor reaches speed. The time intervals are the periods used for the linear least square calculation of sedimentation coefficient. Runs 2003 to 2010 are at 60,000 rpm. The rest are at 52,000 rpm.

Run type	Peak[a] $s_{20,w}$	Peak[a] $\bar{s}_{20,w}$	Time
			min
Zone			
2003	7.49 ± 0.08	7.82 ± 0.04	0–30
2006	7.54 ± 0.08	7.57 ± 0.04	0–30
2013	7.76 ± 0.06	7.85 ± 0.12	0–30
Active enzyme			
2009		7.23 ± 0.32	2–24
		11.32 ± 0.14	26–32
2011		7.89 ± 0.19	9–43
Zone + additives			
2007 20 mM asparagine		7.37 ± 0.03	0–12
		11.70 ± 0.29	14–30
2008 20 mM aspartate	7.45 ± 0.06	7.51 ± 0.04	0–30
2010 20 mM asparagine		7.02 ± 0.23	0–12
		11.45 ± 0.43	14–26
2014 20 mM asparagine		8.14 ± 0.15	0–26
		12.84 ± 0.95	22–36
2015 100 mM asparagine		10.13 ± 0.34	0–18
		17.37 ± 1.80	20–26
2016 100 mM glutamine		10.85 ± 0.22	0–14
		12.85 ± 0.40	18–26

[a] Peak $s_{20,w}$ values are calculated from the migration of the peak absorbance. The $\bar{s}_{20,w}$ values are calculated from the migration of the midpoint of the equivalent boundary.

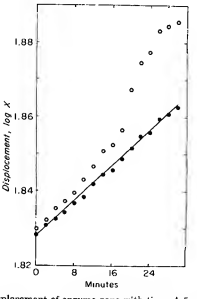

FIG. 3. Displacement of enzyme zone with time. A 5-μl zone of 1.2 mg/ml of enzyme was sedimented in 0.01 M sodium phosphate buffer (pH 7.4) containing 0.2 M NaCl, 3 mM sodium azide, and 3% sucrose. Scans were taken at 280 nm every 2 min after the rotor reached 52,000 rpm. *Closed circles* are a plot of the log of the X displacement of midpoint of the equivalent boundary of the zone sedimented in buffer alone. The *line* represents linear least squares calculation through these points. $s_{20,w}$ was 7.85 ± 0.12 S. *Open circles* are from an identical experiment in buffer containing 100 mM asparagine.

ing its distribution in the animal. The maximal concentrations in plasma during treatment of animals is only about 0.06 mg/ml. Therefore, we developed an active enzyme sedimentation method to test whether association also occurred at low protein concentrations. In this technique, 5 μl of enzyme was simultaneously layered into a double sector cell containing buffer with 10 mM asparagine in one sector and 10 mM aspartic acid in the other. As the zone of enzyme sedimented through the asparagine, part of the substrate was hydrolyzed to aspartic acid which has a lower absorbance at 210 to 233 nm. This absorbance difference has been used for asparaginase assays (10). Time difference patterns of scans from a typical experiment are shown in Fig. 5. The patterns are quite noisy due to the low light intensity at 226 nm, but the figure clearly shows a band moving towards the bottom of the cell in these successive time difference scans. Three of these experiments with initial enzyme concentration of 0.11, 0.12, and 0.37 mg/ml gave $s_{20,w}$ values by equivalent boundary calculations of 7.8 ± 0.5, 7.3 ± 1.5, and 7.5 ± 1.1 S. These experiments indicate that association does not occur under these conditions.

Comparison to Other Enzymes—Table IV compares the *Pseudomonas 7A* glutaminase-asparaginase with several other asparaginases. The divergence of amino acid compositions (11) is given in the top part of the table. The numbers which are given in the Table are calculated from Equation 1,

$$D = 100 \sum_{i=1}^{22} (X_{iA} - X_{iB})^2]^{1/2} \quad (1)$$

where X_{iA} is the mole fraction of amino acid i in protein A and X_{iB} is the mole fraction of the same amino acid in protein B. The numbers in the table lie in the range of homologous proteins (11) with the possible exception of the guinea pig serum enzyme. Clearly, similarity of amino acid composition, does not dictate the plasma half-life. The most similar protein in composition to the *Pseudomonas 7A* enzyme studied here has the shortest half-life.

It has recently been suggested that plasma half-life may be related to the hydrophobicity of the proteins (12) as calculated by the method of Bigelow (13). The last row of the table gives

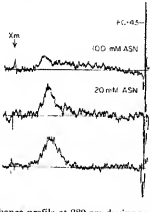

FIG. 4. Absorbance profile at 280 nm during zone sedimentation in different solvents. A 5-μl zone of 1.2 mg/ml of enzyme was sedimented in 0.01 M sodium phosphate buffer (pH 7.4) containing 0.2 M NaCl, 3 mM sodium azide, and 3% sucrose. Each scan was obtained 26 min after the rotor reached 52,000 rpm. The *lower pattern* is from an experiment in buffer alone, the *middle pattern* in buffer containing 20 mM asparagine, and the *upper pattern* from an identical experiment in buffer containing 100 mM asparagine. X_m is the meniscus, and *FC-43* is the position of the buffer-oil interface. *Horizontal lines* are drawn at zero absorbance difference.

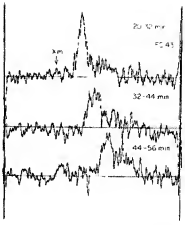

FIG. 5. Time difference profiles of asparagine absorbance at 226 nm. A 5-μl zone of 0.11 mg of enzyme/ml was simultaneously layered into sectors containing 0.01 M sodium phosphate buffer (pH 7.4) containing 0.2 M NaCl, 3% sucrose, and either 10 mM asparagine or aspartic acid. The absorbance of the sectors was scanned every 4 min after the rotor reached 52,000 rpm. The top to bottom patterns are the difference spectrum of the asparagine absorbance at 3 consecutive 12-min intervals beginning at 20 min after the rotor reached speed. X_m is the approximate meniscus location and *FC-43* is oil interface at the cell base.

this hydrophobicity calculation in kilocalories/residue. The correlation of two variables, S and P, is given by Equation 2 (14).

$$C = \frac{\sum SiPi - (\sum Si)(\sum Pi)/N}{[(\sum S_i^2 - (\sum Si)^2/N)(\sum P_i^2 - (\sum Fi)^2/N)]^{1/2}} \quad (2)$$

The correlation, C, varies between +1 and −1, $C = 0$ indicating no correlation of the two variables S and P. For the enzymes of Table IV the correlation of plasma half-life and hydrophobicity was 0.34. If the guinea pig enzyme is omitted from the calculation, the correlation becomes −0.81. Neither correlation is statistically significant.

Previously we have shown a correlation between prolongation of half-life by succinylation of *Acinetobacter* glutaminase-asparaginase and resistance to digestion by trypsin (20). Utilizing the same method, the rate of inactivation by 10 and 20 μg/ml of trypsin was greatest with *Escherichia coli* asparaginase, intermediate with the *Pseudomonas 7A* enzyme and slowest with the *Acinetobacter* enzyme. Thus, the susceptibility to this type of proteolysis does not correlate with half-life.

DISCUSSION

As with other asparaginases we have examined, the catalytic activity of the *Pseudomonas 7A* enzyme is associated with the tetramer and, perhaps, higher polymers. No smaller species were seen. Active polymeric forms of other enzymes have been described but have been assumed to be artifacts of purification or lyophilization (3, 21). *Pseudomonas 7A* glutaminase-asparaginase, as far as we are aware, is the first pure enzyme which has been shown to polymerize in the presence of substrate (22).

Active enzyme sedimentation of asparaginase and glutaminase enzymes in asparagine and glutamine as substrates offers advantages over the use of pseudosubstrates such as 5-diazo-4-oxonorvaline or 6-diazo-5-oxonorleucine. The pseudosubstrates

TABLE IV

Comparison of Asparaginases from Several Sources

Property	Enzyme[a]					
	GA7A	AGA	S. Marc.	Er. cart.	E.C.-2	G.P.S.
Amino acid[b] divergence						
GA7A	0.00	4.10	5.70	6.84	7.84	10.39
AGA	4.10	0.00	5.56	6.71	7.41	11.23
S. Marc.	5.70	5.56	0.00	5.69	5.65	10.30
Er. cart.	6.84	6.71	5.69	0.00	6.69	9.88
E.C.-2	7.84	7.41	5.65	6.69	0.00	13.03
G.P.S.	10.39	11.23	10.30	9.88	13.03	0.00
Plasma half-life in mice (h)	13	1	7	4	4	24
Hydrophobicity[c]	0.994	1.061	1.025	1.065	1.015	1.098

[a] Abbreviations and references for amino acid and half-life data: GA-7A, *Pseudomonas 7A* glutaminase-asparaginase (15); AGA, *Acinetobacter glutaminasificans* glutaminase-asparaginase (3); S. marc. *Serratia marcescens* asparaginase (16); Er. Cart., *Erwinia carotovora* asparaginase (17); E.C.-2, *Escherichia coli* asparaginase (Bayer) (18), G.P.S. guinea pig serum asparaginase (19).

[b] Amino acid compositions from the original reports were used due to discrepancies in the review on asparaginases (16). Divergence calculated from Equation 1 of the text.

[c] kcal/residue, calculated by the method of Bigelow (13).

are poor substrates and may inhibit the enzyme. With the real substrates much lower enzyme concentrations may be employed. However, there are several disadvantages encountered in the use of asparagine or glutamine as substrate. First, the absorbance change upon hydrolysis is small and occurs at wavelengths where protein absorbance is high. Thus, anomalous absorption patterns still occur and require the use of a centerpiece which allows simultaneous layering of the protein zone into each sector (2). Due to high absorbance and low lamp intensity at the 210 to 233 nm range, the signal/noise ratio becomes high. This makes the detection of anomalous sedimentation behavior very difficult, and also leads to large uncertainties in the calculation of sedimentation coefficients. In spite of the disadvantages and difficulty of the experiments, it is helpful to use this method to verify the conclusions made with the pseudosubstrates.

Roberts has shown that the glutaminase-asparaginase from *Pseudomonas 7A* has an isoelectric point similar to *Escherichia coli* asparaginase and that it is not a glycoprotein (1). This paper shows that the molecular weight of the subunit is very similar to that found for other antitumor asparaginase and glutaminase enzymes (3, 16). The enzyme is a tetramer as determined by the molecular weights of native enzyme and subunits. Aside from the *Serratia* enzyme (12), the native protein is quite similar in molecular weight and subunit structure to other bacterial asparaginases (16). A unique property of the enzyme from *Pseudomonas 7A* is its gradual polymerization in the zone sedimentation velocity experiments at high protein concentration (greater than 1 mg/ml) but only in the presence of high substrate levels. This phenomenon was not seen during gel filtration, or zone sedimentation of 0.1 to 0.4 mg/ml of protein in buffers containing asparagine. These lower enzyme concentrations are closer to the maximal levels measured in plasma during enzyme therapy. Therefore, this polymerization may not be occurring *in vivo*. Further studies are needed to test whether the enzyme polymerizes in the presence of plasma proteins or other blood components.

Pseudomonas 7A glutaminase-asparaginase has a much longer plasma half-life than other bacterial asparaginase enzymes (16). Very little is known about the chemical and physical characteristics that effect the rate of clearance of proteins from the circulation. Prolongation of the half-life can be achieved by chemical modification or coupling of glycoproteins to some of the antitumor asparaginase and glutaminase enzymes (20, 23–25). The availability of *Pseudomonas 7A* glutaminase-asparaginase provides a further opportunity to study this process. Examination of the correlation between amino acid similarity and plasma half-life as presented in Table IV does not help to resolve the question. Similarly, the hydrophobicity index of Bigelow (13) does not correlate significantly with plasma half-life. However, when only the bacterial enzymes are considered, a suggestive negative correlation appears, although it is not statistically significant. Holcenberg and Ericsson have shown that the NH_2-terminal portion of *Acinetobacter* glutaminase-asparaginase is homologous with that reported for *E. coli* asparaginase (26). A statistical analysis of aligned sequences of the bacterial enzymes might be fruitful in the explication of the requirements for long plasma half-lives of these proteins; however, it is more likely that the specific surface characteristics of the proteins must be analyzed to yield an unambiguous answer.

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